

Gastrointestinal Hyperplasia with Altered Expression of DNA Polymerase β

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Abstract

Background: Altered expression of DNA polymerase β (Pol β) has been documented in a large percentage of human tumors. However, tumor prevalence or predisposition resulting from Pol β over-expression has not yet been evaluated in a mouse model.

Methodology/Principal Findings: We have recently developed a novel transgenic mouse model that over-expresses Pol β . These mice present with an elevated incidence of spontaneous histologic lesions, including cataracts, hyperplasia of Brunner's gland and mucosal hyperplasia in the duodenum. In addition, osteogenic tumors in mice tails, such as osteoma and osteosarcoma were detected. This is the first report of elevated tumor incidence in a mouse model of Pol β over-expression. These findings prompted an evaluation of human gastrointestinal tumors with regard to Pol β expression. We observed elevated expression of Pol β in stomach adenomas and thyroid follicular carcinomas, but reduced Pol β expression in esophageal adenocarcinomas and squamous carcinomas.

Conclusions/Significance: These data support the hypothesis that balanced and proficient base excision repair protein expression and base excision repair capacity is required for genome stability and protection from hyperplasia and tumor formation.

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Introduction

Increasing evidence is emerging that a large percentage of human tumors have elevated expression of DNA polymerase β (Pol β) [1] and in many cases, mutations within the Pol β coding region results in over-expression of dysfunctional Pol β proteins [2]. High levels of Pol β expression have been demonstrated in several human cancers and tumor cell lines [3–6]. Specifically, elevated Pol β expression is observed in esophageal cancer [7], colorectal cancer [8] and pancreatic cancer [9]. Ectopic Pol β expression in human cancer cells is associated with aneuploidy, abnormal localization of centrosome-associated gamma tubulin protein expression during mitosis, increased microsatellite instability [8,10] and is found to promote tumorigenesis in immuno-

deficient nude mice [4,5]. Recently, infection by several viruses associated with elevated cancer incidence, including chronic myelogenous leukemia (CML) [11], human papillomavirus 16 (HPV16) [12] and Epstein-Barr virus (EBV) [13], has been shown to induce the expression of Pol β to elevated levels. Furthermore, approximately 30% of human cancers express mutant or aberrant forms of Pol β proteins [2,14–16], leading to genomic instability and possibly conferring a mutator phenotype to cells [3,17,18]. Taken together, current evidence indicates an imbalance in Pol β expression, either increased or decreased, leads to functional deficiency of the base excision repair pathway and promotes genomic instability [3,17,18].

As a key enzyme in the base excision repair (BER) pathway, Pol β is essential for the efficient repair of DNA lesions damaged by

endogenous and exogenous genotoxins [19]. Once the base lesion is removed and the DNA backbone is hydrolyzed by the concerted action of a lesion-specific DNA glycosylase such as Methyladenine DNA Glycosylase (MPG) and Apurinic/apyrimidinic endonuclease 1 (APE1), the resulting single-nucleotide gap is 'tailed' by the 5'dRP lyase activity of Pol β and subsequently, Pol β adds a nucleotide to fill the gap. Repair is then completed by the XRCC1/LigIII α heterodimer [20]. BER is severely attenuated in the absence of Pol β , leading to an increase in cellular sensitivity to several genotoxins [21–26], increased spontaneous and damage-induced mutations and genome rearrangements in knockout (KO) or knockdown (KD) cells [17,27,28] and KO (+/–) mice [29]. However, complete KO (–/–) is lethal in mice just after birth [30,31] preventing detailed analysis of Pol β deficiency beyond embryo development where it is found that Pol β KO neurons die by p53-dependent apoptosis [32] resulting in an increase in mutation frequency in the remaining embryonic tissue [33].

Pol β is involved in many essential protein-protein interactions among the various BER proteins [20], yet some Pol β binding proteins suggest additional functions outside of BER, as evidenced by an interaction with the telomere protein TRF2 [34], the ATM binding protein ATMIN (ASCIZ) [35,36], the 9-1-1 checkpoint complex [37], the histone acetyltransferase MYST2 [38] or the transcription factor TAF1D (JOSD3, MGC5306) [39]. The functional significance of many of these potential interactions has yet to be revealed. However, Pol β was identified by ChIP analysis as a component of the telomere protein complex [40], a role that is likely related to its interaction with TRF2 [34]. These significant protein-protein interactions and the role of Pol β in BER or other DNA metabolic functions can clearly be impacted by protein expression changes that would disrupt complex formation.

In addition to control via transcription or translation, Pol β is also regulated post-translationally via acetylation [41], methylation [42,43] and ubiquitylation [44,45]. These varied modes of Pol β regulation can impact not only Pol β function directly, but changes in expression or in specific post-translational modifications (PTM) can alter function (loss of stability or loss of function due to PTM) or can impact complex formation (loss of protein-protein interactions due to PTM) [20] and lead to repair defects even when Pol β is expressed at high levels.

Transgenic mice with over-expression of the Flag-Pol β transgene were developed here to study the consequences of this effect on imbalanced base excision repair and carcinogenesis. As part of the characterization of this animal model, we report the age-associated histopathological changes present in two-year old Pol β transgenic mice. We find that mice over-expressing Pol β develop Brunner's gland hyperplasia, mucosal hyperplasia in the duodenum and osteogenic tumors in the tail. This was the impetus for an analysis of Pol β expression in relevant human gastrointestinal tumors and the surrounding normal tissue. Paradoxically, we find that whereas stomach adenocarcinoma and thyroid follicular carcinoma present with slightly elevated expression of Pol β , both esophageal squamous carcinoma and esophageal adenocarcinoma show a significant decrease in Pol β expression, compared with surrounding pathologically normal tissue. Overall, these studies support the hypothesis that balanced and proficient BER protein expression and BER capacity is required for genome stability and protection from hyperplasia and tumor formation.

Results

It is our hypothesis that altered expression of Pol β and the resulting imbalance in BER can predispose to tumor formation.

To test this hypothesis, we analyzed transgenic mice that present with elevated expression of Pol β [46]. These Pol β transgenic (Tg) mice express Flag-tagged Pol β (TetOp-Flag-Pol β -tTA) and were described previously [46]. In this present study, the Tg mice were crossed >5 generations to the C57Bl/6 strain. In rare cases,

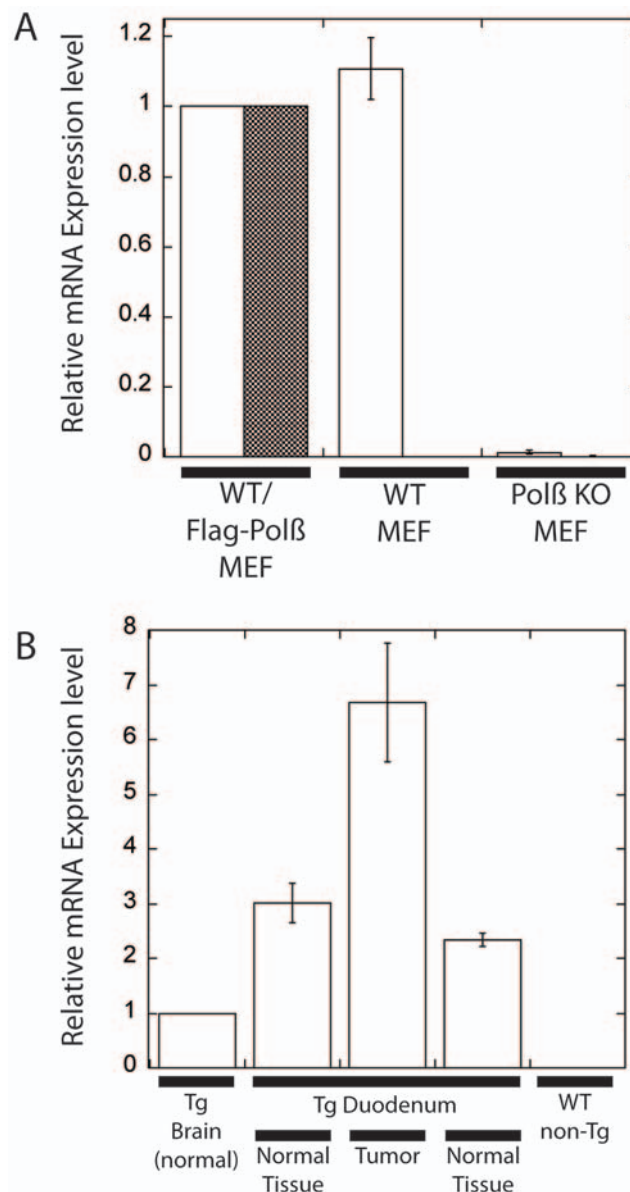


Figure 1. Expression of Flag-Pol β in MEFs and tissues from Pol β Tg mice. (A) Specificity of mouse and human qRT-PCR analysis for Pol β expression: RNA was isolated from WT and Pol β KO MEFs and MEFs expressing the Flag-Pol β transgene, as described in the Methods section. The relative level of expression of both the mouse (open bars) and human (filled bars) Pol β mRNA (normalized to mouse β -actin) was determined using mouse and human specific Taqman assays. Expression across samples was normalized to the expression level in the WT/Flag-Pol β MEF sample. (B) Expression of the human Pol β transgene in mouse tissues and tumors: RNA was isolated from the sample indicated in the plot, as described in the Methods section. The relative level of expression of human Pol β mRNA (open bars; normalized to mouse β -actin) was determined using human specific Taqman assays as in panel A. Expression across samples was normalized to the expression level in the Tg Brain sample. doi:10.1371/journal.pone.0006493.g001

repression of transgene expression has been reported, depending on the transgene, the promoter used for expression and its location [47–49]. To verify that expression of the Flag-Pol β transgene was maintained and was not epigenetically silenced during the backcrosses from the original Tg strain to C57BL/6 mice, expression was verified by qRT-PCR of RNA purified from tissue isolated by laser-capture microdissection. The Pol β human transgene used herein has 90.8% sequence identity to the mouse Pol β cDNA (not shown) so we first verified that the Taqman gene expression assays were specific for each mRNA. RNA was isolated from wild-type (WT) mouse embryonic fibroblasts (MEFs), Pol β KO MEFs and WT MEFs that express the Flag-Pol β transgene used in this study. The relative level of expression of either the mouse or human Pol β mRNA was determined for each sample and normalized to the expression of mouse β -actin using the $\Delta\Delta C_T$ protocol, as described in the Methods section. As shown in Figure 1A, the expression of both mouse and human Pol β was normalized to the expression level in the transgenic cell line WT/Flag-Pol β . A similar level of expression of mouse Pol β was observed in the WT MEF cell line but no expression of mouse Pol β was detected in the Pol β KO cell line, as expected [21,22].

Similarly, the expression of the human Pol β transgene was only detected in the WT/Flag-Pol β MEF cells (filled bar, Figure 1A) with no detectable expression in either the WT MEF cells or the Pol β KO MEF cells. This analysis demonstrates the specificity of both the mouse and human Taqman gene expression assays. It should be noted that we used this approach to validate the specificity of a second Taqman gene expression assay for human Pol β (Hs00160263_m1). However, this assay cross-reacted with the mouse mRNA and was not used.

Using the validated gene expression assays and the $\Delta\Delta C_T$ protocol as described above and in the Methods section, we next determined if the human Flag-Pol β transgene was expressed in the cells of the tissues of interest and most importantly, in the tumors. Using laser-capture microdissection, we isolated and purified RNA from the brain (not shown) and the normal and tumor samples described in Figure 2, as well as from cells from a non-transgenic (non-Tg) mouse. Previous studies (not shown) suggested that the expression of the Flag-Pol β transgene in the brain was low but detectable [46] and so the relative quantitation of Flag-Pol β expression was normalized to the level of expression in the brain (Figure 1B). As shown, the level of expression in normal duodenum tissue was slightly elevated as compared to the

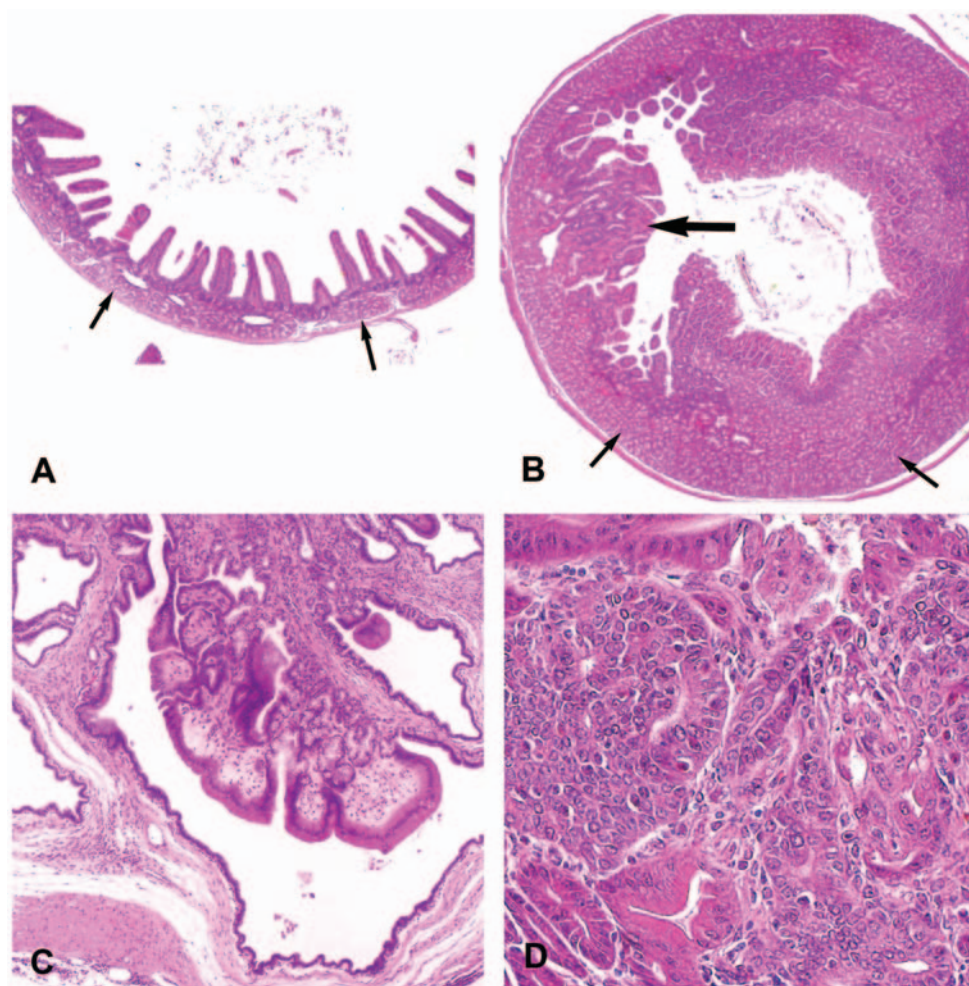


Figure 2. Representative photomicrographs (H & E stain) of duodenal changes in Pol β Tg mice. (A) Normal duodenum. Note normal Brunner's glands (arrows) (magnification $\times 20$). (B) Diffuse hyperplasia of Brunner's glands (small arrows) and duodenal crypt epithelium (large arrow). Note markedly increased mucosal thickness due to glandular hyperplasia, compared to panel A (magnification $\times 20$). (C) Cystic dilatation of mucosal crypts and Brunner's glands with displacement of cystic glands into the tunica muscularis (magnification $\times 200$). (D) Focal proliferation of dysplastic glands in a mouse diagnosed with duodenal adenoma (magnification $\times 400$).
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brain, similar to that observed in our previous analysis of this Tg mouse [46]. However, expression in the hyperplastic duodenum was approximately 7-fold higher than the brain and 2- to 3-fold higher than the normal duodenum tissue. These studies therefore confirm that the Flag-Pol β transgene is expressed at elevated levels depending on the tissue and most importantly, the transgene expression appears to be further elevated in the tumors (Figure 1B). Further, these results suggest that the expression of the Flag-Pol β transgene is elevated in these mice similar to that reported earlier [46].

Table 1. Incidence of macroscopic findings observed in DNA polymerase β Tg mice.

Organ	Macroscopic Finding	Frequency (%)*	
		Male (n = 15)	Female (n = 21)
Abdominal cavity	Fluid	13.3	19.0
	Mass/Nodule	6.7	0.0
Heart	Enlarged	13.3	0.0
	Soft	0.0	4.8
Salivary gland	Atrophy	0.0	28.6
	Mass/Nodule	0.0	9.5
Liver	Cysts	6.7	28.6
	Enlarged	0.0	9.5
	Mottled	20.0	9.5
	Mass/Nodule	20.0	9.5
Duodenum	Thickened, red	0.0	14.3
	Mass/Nodule	13.3	23.8
Jejunum	Mass	6.7	4.8
Ileum	Mass	0.0	4.8
Colon	Mass	6.7	0.0
Adrenal	Enlarged	6.7	4.8
Pituitary gland	Dark, cystic	0.0	4.8
Spleen	Enlarged	6.7	38.0
	White Foci	6.7	0.0
	Small/Atrophy	6.7	4.8
	Mass/Nodule	6.7	4.8
Thymus	Enlarged	0.0	4.8
Mesenteric Lymph Node	Enlarged	40.0	4.8
	Mass/Nodule	0.0	4.8
Other Lymph Node	Enlarged	20.0	38.1
Testis	Dark red	6.7	–
Seminal vesicle	Black, cystic	6.7	–
	Enlarged	20.0	–
Prostate	Enlarged	6.7	–
Ovary	Dark red	–	19.0
Uterus	Dilated	–	4.8
Lung	Pale	6.7	0.0
Ear	Ulcer	0.0	4.8
Eye	Lens, white	100	100
Tail	Mass/Nodule	33.3	14.3

*Frequency defined as the number of animals with the lesion divided by the number of animals with the tissue examined macroscopically, multiplied by 100.

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We therefore evaluated the spectrum of pathological lesions in a cohort of our Pol β Tg mice at 2 years of age. Macroscopic lesions are summarized in Table 1 and non-neoplastic and neoplastic lesions are summarized in Tables 2 through 4. Morphological characteristics of some of the main lesions are as follows in the sections below.

Proliferative lesions of the duodenum

Hyperplasia of Brunner's glands and hyperplasia of the duodenal crypt epithelium resulting in markedly thickened duodenal mucosa was detected in four of 15 male mice (26.7%) and ten of 21 female mice (47.6%) (compare Figure 2A and 2B). Proliferative lesions, especially around Vater's papilla, were

Table 2. Incidence of non-neoplastic lesions observed in DNA polymerase β Tg mice.

Organ	Macroscopic Finding	Frequency (%)*	
		Male (n = 15)	Female (n = 21)
Heart	Polyarteritis	6.7	0.0
Salivary gland	Mononuclear cell, infiltration	93.3	45.0
Duodenum	Erosion	0.0	9.5
	Ectopic pancreas	0.0	4.8
Liver	Altered cell foci	6.7	4.8
	Biliary cyst	6.7	14.3
	Chronic active hepatitis	13.3	0.0
	Erythrophagocytosis	0.0	4.8
	Extramedullary hematopoiesis	6.7	14.3
	Hemorrhage	20.0	4.8
	Hyaline bodies, cytoplasm	6.7	0.0
	Microgranulation	0.0	4.8
	Mononuclear cell, infiltration	53.3	47.6
	Necrosis	13.3	14.3
	Pigmentation	0.0	4.8
	Subcapsular infiltration	0.0	4.8
Gallbladder	Hyaline inclusion	27.3	5.0
Pancreas	Mononuclear cell, infiltration	0.0	19.0
Adrenal gland	Pigmentation	33.3	95.2
Mesenteric Lymph Node	Erythrophagocytosis	0.0	8.3
Other Lymph Node	Plasmacytosis	6.7	0.0
Spleen	Congestion	0.0	4.8
	Extramedullary hematopoiesis	73.3	75.0
Mammary gland	Lactation	–	5.6
Testis	Sperm granuloma	14.3	–
	Hemorrhage	7.1	–
	Tubular, atrophy	7.1	–
Epididymis	Hypospermia	7.7	–
Prostate	Mononuclear cell, infiltration	6.7	
	Polyarteritis	6.7	
Seminal vesicle	Hemorrhage	6.7	

*Frequency defined as the number of animals with the lesion divided by the number of animals with the tissue examined histopathologically, multiplied by 100. Abnormal non-neoplastic changes were not detected in thyroid, parathyroid, thymus, stomach, jejunum, ileum, cecum, colon and pituitary.

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Table 3. Incidence of non-neoplastic lesions observed in DNA polymerase β Tg mice.

Organ	Macroscopic Finding	Frequency (%)*	
		Male (n = 15)	Female (n = 21)
Ovary	Atrophy	–	94.4
	Cyst	–	60.0
	Thrombus	–	5.6
Vagina	Erosion	–	50.0
Lung	Mononuclear cell, infiltration	60.0	57.1
Eye	Cataract	100.0	100.0
Kidney	Basophilic tubules	86.7	80.0
	Glomerular, hyalinization	100.0	85.0
	Glomerular, hypercellularity	20.0	20.0
	Mononuclear cell, infiltration	80.0	85.0
	Tubular, vacuolization	93.3	0.0
	Hyaline droplet	0.0	15.0
	Protein cast	0.0	10.0
Skin	Pelvic, dilatation	0.0	5.0
	Ulcer	0.0	5.0
	Crust	0.0	5.0
	Mononuclear cell, infiltration	0.0	10.0
Brain	Mineralization	53.3	15.8
	Hemosiderin deposition	6.7	0.0
	Lateral ventricle, dilatation	13.3	0.0
	Mononuclear cell, infiltration	0.0	5.3
	Polyarteritis	6.7	0.0
Urinary bladder	Mononuclear cell, infiltration	8.3	38.1
Tail	Chondro-osseous metaplasia	–	33.3

*Frequency defined as the number of animals with the lesion divided by the number of animals with the tissue examined histopathologically, multiplied by 100. Abnormal non-neoplastic changes were not detected in thyroid, parathyroid, thymus, stomach, jejunum, ileum, cecum, colon and pituitary.
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detected with high incidence. Such changes were characterized by diffuse epithelial hyperplasia, altered epithelial differentiation, eosinophilic cytoplasmic inclusions, and herniation/diverticulation (Figure 2C) of the epithelium into the tunica muscularis and serosa. Adenoma of relatively small size, was diagnosed in one of 21 female mice (Figure 2D). Proliferative lesions were often associated with chronic granulomatous inflammation and are similar to duodenal plaques (avillous hyperplasia, duodenum polyp) derived from the crypts of Lieberkuhn [50]. Most of these lesions are regarded as benign hyperplastic lesions, but some may develop dysplasia, a pre-malignant neoplastic lesion.

Osteogenic tumors in tail

Macroscopically, masses or nodules in the tail were detected in 33.3% (5/15) of male and 14.3% (3/21) of female mice. Histopathologically, chondro-osseous metaplasia was detected in one of 3 female mice; osteoma was detected in three of 5 male mice (Figure 3A and 3B); and osteosarcoma was detected in one of 5 male and one of 3 female mice (Figure 3C and 3D). Osteosarcoma was characterized by proliferating spindle tumor cells, associated with presence of irregular, infiltrating trabecular bone. In this study, osteogenic tumors were not detected in any other site besides the tail.

Mature Cataract

Mature cataracts were detected in all mice examined (male; 13/13, female; 21/21), essentially as we described previously [46]. Histopathologically, the lens changes were characterized by degeneration/necrosis (liquefaction), vacuole formation in most of the lens fibers, and irregular proliferation of lens fiber spindle cells, followed by calcification (Figure 4A and 4B). Moreover, lens epithelia with bizarre nuclei and single cell necrosis in the proliferative fiber cells were seen. In all cases, lenticular lesions were diagnosed as a mature type, a final stage of cataract formation.

Renal lesions

A spectrum of renal lesions was detected in almost all animals with glomerular changes, basophilic tubules, and mononuclear cell infiltration occurring in both sexes. Glomerular hyalinization was detected in almost all mice (male; 15/15, female; 17/20) with basement membrane thickening (Figure 4C and 4D). In mild cases, eosinophilic material in glomerular basement membranes was seen, while densely eosinophilic and amorphous deposits in glomeruli were seen in severe cases. In addition, glomerular hypercellularity, consistent with proliferation of mesangial cells, was present in 3 of 15 male and 4 of 20 female mice. Moreover, basophilic tubules observed in the cortex of 13 of 15 male and 16 of 20 female mice. These lesions are representative of glomerulonephritis.

Expression of Pol β in human tumors and surrounding normal tissue

To extend observations in mice, we examined Pol β expression in human gastrointestinal cancers and in surrounding normal epithelial tissues (Figure 5A). Because cancers are more likely to arise in epithelial tissues, we separated scores in epithelial and stromal (subepithelial) areas. There was no significant difference in Pol β immunoreactivity scores between stromal areas in cancerous vs. surrounding non-cancerous tissues. In epithelial tissues, we observed (Figure 5B) elevated expression of Pol β in stomach adenocarcinomas compared with surrounding normal gastric mucosal tissue, although this difference was not significant ($p = 0.19$). Compared with paired normal surrounding tissues, however, there was a significant increase in Pol β in thyroid follicular carcinomas ($p = 0.02$), but significantly reduced Pol β expression in esophageal adenocarcinomas ($p = 0.04$) and squamous carcinomas ($p = 0.005$).

Discussion

BER proteins require a finely tuned balance of expression to ensure complete repair of many mutagenic or genome destabilizing base lesions [24,46,51–54]. Altered expression or mutations in BER proteins such as Pol β that impact function or protein-protein interactions can predispose to sensitivity to genotoxins [22], an increase in genome alterations [17], mutations [27] and tumor formation [55]. In total, cellular, epidemiological and pathological analyses suggested a correlation between several human cancers and Pol β mutations and/or expression changes.

In cell-based studies, alteration in expression of Pol β impacts BER capacity and manifests as a genome destabilizing phenotype, consistent with the observation that greater than 30% of human tumors have elevated expression of Pol β [1] and in a separate study it was revealed that greater than 30% of human tumors express mutant forms of Pol β [2]. Only a few animal models with altered Pol β expression have been characterized to study how alterations in Pol β expression might impact tumor formation in

Table 4. Incidence of non-neoplastic & neoplastic proliferative lesions in DNA polymerase β Tg mice.

Organ	Lesion	Frequency (%)*		Organ	Lesion	Frequency (%)*	
		Male	Female			Male	Female
Salivary gland	Lymphoid hyperplasia	0.0	10.0	Other Lymph Node	Lymphoid hyperplasia	5.6	3.8
	Malignant lymphoma	0.0	15.0		Malignant lymphoma	11.1	15.4
Forestomach	Squamous hyperplasia	0.0	5.0		Histiocytic sarcoma	0.0	30.8
Duodenum	Brunner's gland/Mucosa Hyperplasia,	26.7	47.6	Spleen	Lymphoid hyperplasia	13.3	25.0
					Malignant lymphoma	6.7	5.0
					Histiocytic sarcoma	6.7	5.0
	Adenoma	0.0	4.8	Thymus	Malignant lymphoma	0.0	50.0
	Malignant lymphoma	6.7	4.8	Skin	Lymphoid hyperplasia	6.7	5.0
Jejunum	Malignant lymphoma	6.7	10.0		Squamous, hyperplasia	0.0	5.0
Liver	Adenoma	13.3	0.0	Ovary	Malignant lymphoma	–	5.6
	Histiocytic sarcoma	13.3	14.3		Histiocytic sarcoma	–	5.6
Adrenal gland	Cortical hyperplasia	16.7	9.5	Uterus	Endometrial, hyperplasia	–	31.6
	Cortical spindle cell hyperplasia	0.0	90.5		Lymphoid hyperplasia	6.7	9.5
Thyroid	Follicular cell, hyperplasia	0.0	18.8	Lung	Malignant lymphoma	0.0	9.5
	Follicular cell adenoma	0.0	6.3		Histiocytic sarcoma	6.7	4.8
Pituitary gland	Hyperplasia	0.0	25.0		Bronchiolar-alveolar adenoma	20.0	0.0
Mesenteric Lymph Node	Lymphoid hyperplasia	9.0	0.0	Kidney	Malignant lymphoma	0.0	5.0
	Malignant lymphoma	63.6	58.3		Osteoma	60.0	0.0
	Histiocytic sarcoma	9.0	25.0		Osteosarcoma	20.0	33.3

*Frequency defined as the number of animals with the lesion divided by the number of animals with the tissue examined histopathologically, multiplied by 100. Data derived from 15 males and 21 females except where noted. No neoplastic changes were detected in gallbladder, parathyroid, thymus, ileum, cecum, colon, pancreas, brain, eye, urinary bladder, testis, epididymis, prostate, seminal vesicle, oviduct, vagina, and mammary gland.

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the whole animal. Mice with a partial deficiency in Pol β expression (Pol β heterozygote mice) have an elevated mutant frequency in male germ cells [27] and a small increase in the incidence of lymphoid hyperplasia and adenocarcinoma [56]. Mice expressing a truncated form of Pol β (polbeta Δ) in mammary glands showed an elevated incidence of tumor formation [55]. This mutant of Pol β is a splice-variant missing amino acids 208–236 [57] however, this variant is not cancer specific [58]. A second Pol β Tg mouse was developed but this model only expresses Pol β in the thymus and elevated tumor formation was not observed [59].

The development of the Pol β transgenic mice used in this study and the Pol β expression pattern was described previously [46]. The Pol β Tg mice were backcrossed onto a C57BL background, a strain widely used for development of transgenic mice and gene-targeting experiments [60]. The majority of non-neoplastic and neoplastic lesions observed in the Pol β Tg mice were considered spontaneous and age-related as previously reported in C57BL and other mouse strains [50,60–63]. The more commonly occurring spontaneous lesions in C57BL/6 (e.g., lymphoma and histiocytic sarcoma) were not increased in the Pol β Tg mice. Lesions unique to the Pol β Tg mice were found in the duodenum, tail, eye, and kidney. The increased incidence (100%) of lesions in the eye (cataract) was described earlier [46].

The 39% incidence of proliferative duodenal lesions in the Pol β Tg mice is considerably higher than previously reported incidences of 4% [63] and 21% [64] in aged C57BL mice. Other spontaneous plaque-like lesions or polyposis in the pyloric area of the glandular stomach have been observed in C57BL/Ncr \times 129/

SvTer (B6,129) mice, 129/SvTer mice, Ahr-null mice, TGF β -1 heterozygous mice, Smad4 heterozygous mice, CYP1A2-null mice, and B6C3F1 mice [65–70]. Moreover, C57BL mice have been reported to be very susceptible to duodenal neoplasia following some carcinogen treatments [71]. A majority of the non-neoplastic lesions are considered spontaneous age-related and have been reported in C57BL/6 and other mouse strains. The incidence of the non-neoplastic lesions was not increased in the DNA Polymerase β over-expressing Tg mice. In particular, mononuclear cell infiltration in all of the organs (mainly lymphocytes), ovarian cystic lesions and atrophy, and liver lesions are very common lesions in aged C57BL/6 mice. As well, there was no gender differences. Therefore, Pol β Tg mice might also be suitable models for duodenal carcinogenicity following treatment with appropriate carcinogens.

Duodenal epithelial tumors that develop from the intestinal type or the pancreaticobiliary type mucosa of Vater's papilla [72,73] are relatively rare tumors in humans; the incidence rate of adenoma is 0.04–0.62% and that of carcinoma is 0.2% in postmortem or autopsy studies [74]. Molecular alterations in these duodenal lesions are similar to those of colorectal tumors and include K-ras mutation and the overexpression of p53, p21/Waf1, p16, and/or APC [72,73,75]. Mice which carry a mutation in the Apc gene have multiple neoplastic lesions in duodenum (42%), jejunum (38%), stomach (25%), ileum (15%), and colon (8%) [76]. It is not known if altered expression of the above-mentioned genes might be related to the pathogenesis of duodenal lesions in Pol β Tg mice. Recently, it was reported that APC directly inhibits BER [77]. It is therefore possible that APC functions to regulate BER,

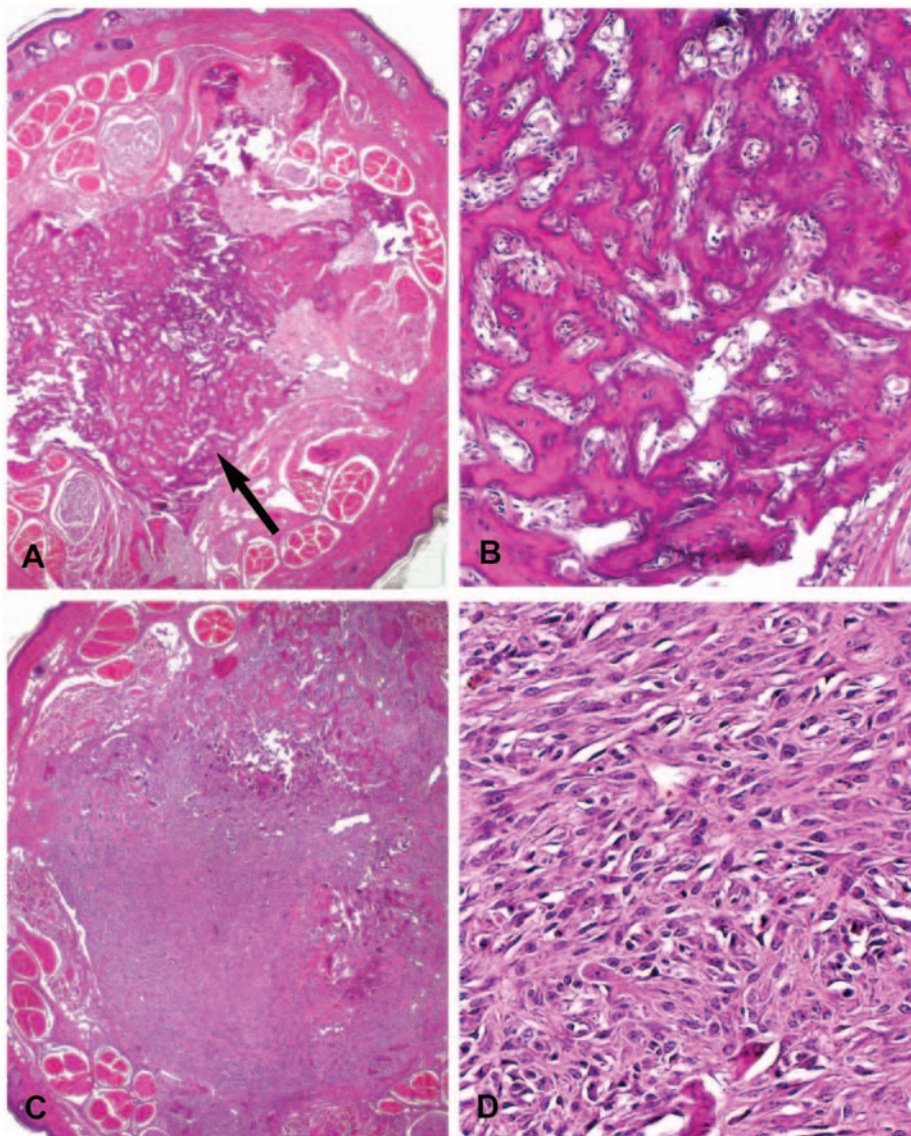


Figure 3. Osteogenic tumors of tails in Pol β Tg mice (H & E stain). (A) Osteoma (arrow) in the central area of tail (magnification $\times 20$, Decalcified). (B) Higher magnification of panel A. Irregular trabecular formation by spindle tumor cells (magnification $\times 200$). (C) Osteosarcoma. The tumor occupied almost the entire subcutaneous area of the tail (magnification $\times 20$). (D) Spindle tumor cells resemble fibroblastic mesenchymal cells and proliferated with production of a small amount of osteoid deposition (magnification $\times 400$).
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suggesting that Pol β over-expression may lead to a similar phenotype as APC deficiency.

Spontaneous occurrence of osteogenic tumors is extremely rare in rats and mice, and there are only a few reports published with incidences of 0.1 to 2% in mice and 0 to 4% in rats [50,78–83]. Therefore, the incidence of osteogenic tumors in Pol β Tg mice is considerably higher than incidences cited in the literature. While the vertebral column including caudal vertebrae (tail) has previously been noted as a common site of osteogenic tumors in mice [81], most reports do not detail the specific sites of osteogenic bone tumors.

Glomerulonephritis and related renal lesions are common findings in C57BL/6 mice [60]. However, it is not clear whether the incidence of glomerulonephritis in the Pol β model is higher than that in C57BL/6 mice due to lack of available data in two-year old C57BL/6 mice.

A higher level of Pol β expression has been found in human intestinal adenocarcinoma than in other organs tumors [3,84]. In this

study, we observed a variable outcome in that ESC and EA presented with decreased expression of Pol β whereas SA and TFC showed elevated Pol β expression. In all cases, the efficiency of the DNA repair system (Pol β) might be compromised due to altered PTM or complex formation [84]. Unfortunately, there have been no reports about the relationship between Pol β expression and osteogenic tumors in humans and animals. Bergoglio and colleagues reported that tumor induction could not be seen in a Pol β Tg mouse model with thymus-specific Pol β transgene expression, suggesting that Pol β over-expression is not sufficient to initiate tumorigenesis *in vivo* [59]. In our Pol β Tg mice, over-expression of Pol β in most systemic tissues was confirmed and the degree of over-expression of Pol β in small intestine was shown to be similar to that in lens [46]. Therefore, we believe that the relationship between lesion pathogenesis and over-expression of Pol β might reflect organ specificity.

The relationship between Pol β over-expression and human carcinogenesis remains to be elucidated. Previous reviews have

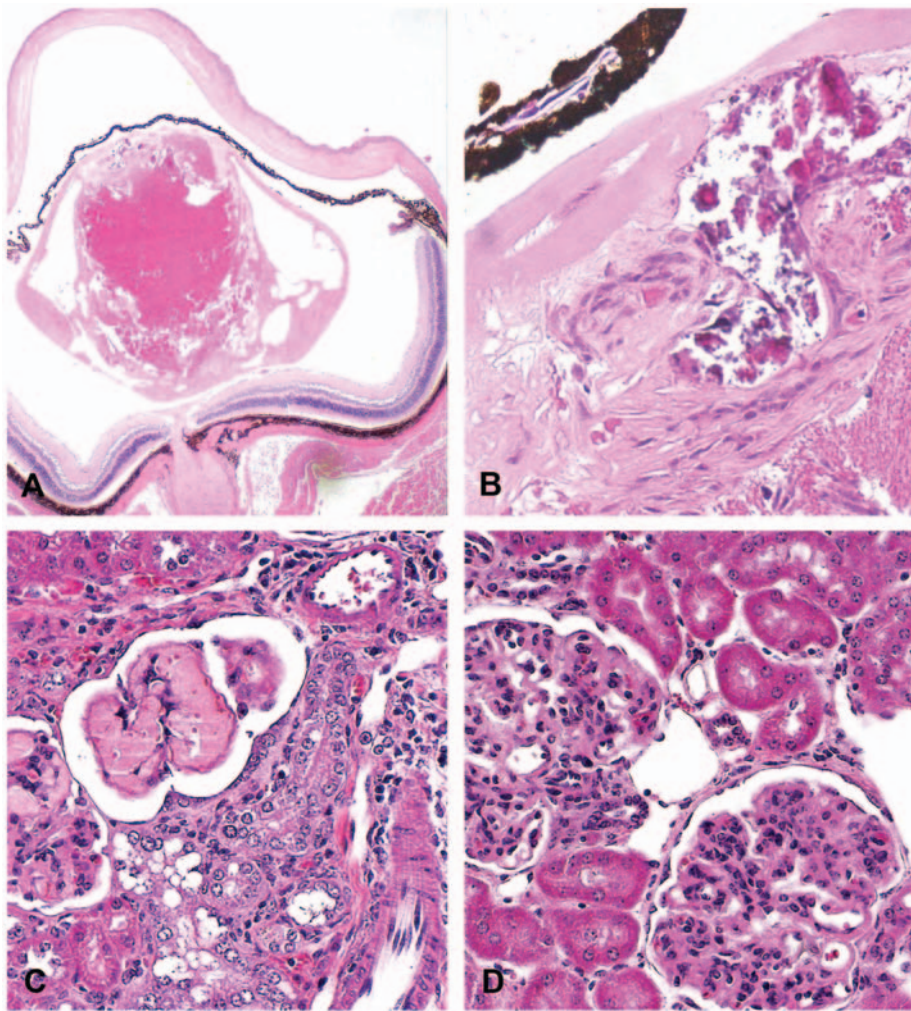


Figure 4. Representative photomicrographs (H & E stain) of lenticular and glomerular damages in Pol β Tg mice. (A) Mature cataract characterized by degeneration/necrosis (liquefaction) and vacuolar formation in lens fibers (magnification $\times 20$). (B) High-magnification of figure a. Irregular proliferation of spindle lens fiber cells without production of normal lens fibers; necrosis and calcification are present (magnification $\times 400$). (C) Glomerular hyalinization with basement membrane thickening of Bowman's capsule, basophilic tubules, and tubular vacuolation in the renal cortex (magnification $\times 400$). (D) Glomerular hypercellularity with basement membrane thickening (magnification $\times 400$).
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reported that greater than 30% of human tumors have elevated expression of Pol β [1] or express dysfunctional Pol β proteins [2]. Additional research is needed to analyze the relationship between Pol β over-expression and intestinal carcinogenesis and the understanding of the potential extrapolations from our model to humans due to over-expression of Pol β . This mouse model might be a useful tool for cancer chemotherapy as well as evaluating the environmental and genetic factors that cooperate with Pol β expression variation to impact hyperplasia and tumor formation.

Materials and Methods

Animals

DNA polymerase Pol β transgenic mice were described previously [46]. Genotyping was as described [46]. These mice (fifteen male and twenty-one female), express Flag-tagged Pol β (TetOp-Flag-pol β -tTA) and were developed in B6SJL-hybrid females and then back-crossed onto C57BL/6 mice [46]. The Pol β expression pattern and the level of over-expression was described previously [46]. These mice over-express Pol β in almost all

organs, including stomach and small intestine [46]. This mouse strain is available from the NIH-sponsored Mutant Mouse Regional Resource Centers (MMRRC) (Strain name: B6.Cg-Tg(TetOp-Polb/tTA)2Sbl/Mmmh, Stock number: 000356-MU). Details for this strain are available at <http://www.mmrcc.org/strains/356/0356.html>. All breeding was at NIH using IACUC and ALAAS approved protocols for the duration of the study. The transgenic mice used in the study were crossed to C57BL/6 (Taconic) for >5 generations, as in the previous study [46]. Each Tg animal is considered a heterozygous Tg mouse in that breeding was only performed using either a transgenic male and C57BL/6 female or a C57BL/6 male and transgenic female. Transgenic mice were never inter-bred. Mice were housed in solid-bottom polycarbonate cages. Filtered room air underwent at least 10 changes per hour. The animal room was maintained at $22 \pm 2^\circ\text{C}$ with $50 \pm 15\%$ relative humidity and a 12-hour light-dark cycle. Irradiated NTP-2000 pelleted feed (Zeigler Bros., Inc., Gardner, PA) and water were available *ad libitum*. Animal handling and husbandry were conducted in accordance with NIH guidelines [85].

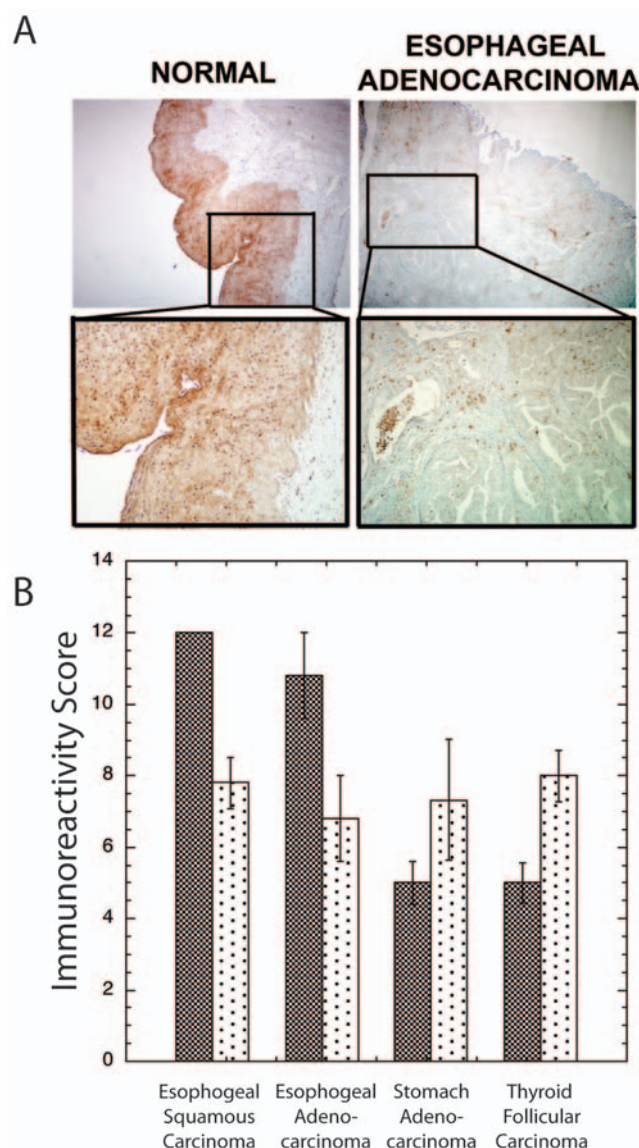


Figure 5. Decreased expression of Pol β in human esophageal adenocarcinoma. (A) Photomicrograph of sections of esophageal adenocarcinoma and esophageal squamous mucosa stained for Pol β expression by immunohistochemistry. Top images reflect magnification $\times 40$ and the inserts depict magnification $\times 100$. (B) Bar graph representing relative expression level of Pol β in various tumors (stippled, open bars) and pathologically normal (grey bars) epithelial tissues. Immunoreactivity Score is the average of 5 different tumor samples each evaluated in two independent analyses. doi:10.1371/journal.pone.0006493.g005

Pathology

Necropsies were performed on all mice after euthanization at the age of 24 months. Euthanasia was by asphyxiation with carbon dioxide and mice were necropsied within 5 min of death. At necropsy, all tissues including masses and macroscopical abnormalities were removed and fixed in 10% neutral buffered formalin. After fixation, the following tissues were trimmed, dehydrated, cleared, and paraffin-embedded: liver, gallbladder, lung, thyroid gland, parathyroid gland, salivary gland, spleen, heart, kidney, stomach, duodenum, jejunum, ileum, cecum, colon, pancreas, mesenteric lymph node, skin, mammary gland, brain, eye, urinary bladder, testis, epididymis, prostate, seminal vesicle, ovary, uterus,

vagina, adrenal gland, and pituitary gland. Other lymph nodes (mediastinal, pancreatic, cervical, and/or perirenal) were examined when macroscopical lesions were detected. In addition, for animals that had macroscopical tail lesions, samples were decalcified prior to routine processing. Five-micron thick sections were mounted onto glass slides, stained with hematoxylin and eosin (H&E), and examined microscopically. The severity of non-neoplastic lesions was graded on a four-point scale of 1 = minimal, 2 = mild, 3 = moderate, and 4 = marked. Histopathology evaluation was performed by two pathologists (KY and AN). Previously published histopathological terminology and diagnostic criteria were used [50,60,63,64].

Laser-Capture Microdissection of Paraffin-Embedded tissue and tumor samples

RNA was isolated from laser capture micro-dissected Formalin-Fixed Paraffin-Embedded (FFPE) tissue and tumor samples using the CellcutTM instrument (Molecular Machines and Industries, Haslett, MI). Two sections (8 μ m) were placed on five polyethylene terephthalate (PET) foil slides. A detailed protocol for staining and the LM process is available on the NIEHS Laser Microdissection Core Facility web site [86,87]. Using the Pure-LinkTM FFPE RNA Isolation Kit (Invitrogen Life Technologies, Carlsbad, CA.), the LM samples and whole section controls were lysed and RNA isolated on the same day.

Quantitative RT-PCR Analysis

Expression of mouse and human Pol β mRNA was measured by quantitative RT-PCR using an Applied Biosystems StepOnePlus system. Briefly, 80,000 MEF cells (WT, WT expressing the Flag-Pol β transgene and Pol β KO) were lysed and reverse transcribed using the Applied Biosystems Taqman[®] Gene Expression Cells-to-CTTM Kit. Each sample was analyzed in triplicate and the results shown are an average of all three analyses. Analysis of mRNA expression was conducted as per the manufacturer ($\Delta\Delta C_T$ method) using Applied Biosystems TaqMan[®] Gene Expression Assays (human POL β : part #4331182, Hs01099715_m1; mouse Pol β : part #4331182, Mm00448234_m1) and normalized to the expression of mouse β -actin (part #4352933E).

For the analysis of the Tg mouse tissue, RNA was extracted as described above and cDNA was synthesized from 30 ng of RNA using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (part #4375575). The cDNA was pre-amplified for 10 cycles using the TaqMan[®] PreAmp Master Mix (part #4391128) and diluted 1:5. The pre-amplified cDNA was next analyzed using the Applied Biosystems TaqMan[®] Gene Expression Assays (human POL β : part #4331182, Hs01099715_m1; mouse Pol β : part #4331182, Mm00448234_m1) and normalized to the expression of mouse β -actin. Expression analysis was determined using the $\Delta\Delta C_T$ protocol as per the manufacturer to determine the relative quantitation of Flag-Pol β expression, as compared to the mouse β -actin among all samples. From the tissue samples, expression was normalized to the level of expression in the brain of Tg mice.

Immunohistochemistry for Pol β in human epithelial tumors

Tissues samples [Esophageal Squamous Carcinoma (ESC), Esophageal Adenocarcinoma (EA), Thyroid Follicular Carcinoma (TFC) and Stomach Adenocarcinoma (SA) and surrounding normal tissue] were obtained through the Tissue and Research pathology Services (TARPS), University of Pittsburgh Cancer Institute. Five cases were examined for each of the types of

carcinoma. Five-micron thick sections of paraffin-embedded tissue on glass slides were either stained with hematoxylin and eosin (H&E) or analyzed for Pol β expression by immunohistochemistry. Labeling was performed on formalin-fixed, paraffin-embedded tissues by incubation with antibodies against Pol β (Abcam, polyclonal, cat#AB53059, diluted 1 in 5000). To ensure even staining and reproducible results, sections were incubated by slow rocking overnight in primary antibody (4°C) using the Antibody Amplifier™ (ProHisto, LLC, Columbia, SC). Following incubation with primary antibody, sections were processed with a rabbit polyclonal EnVision+System-HRP kit (DakoCytomation, Carpinteria, CA) according to the kit protocols. The chromogen was diaminobenzidine and sections were counter stained with 1% methyl green. The negative controls were tissues from Pol β knockout mice, which were negative for staining. Immunohistochemistry was quantified by two independent investigators in a blind fashion as previously described [88]. Cases with a disagreement of both investigators on the immunoreactive score were discussed using a multiheaded microscope until consensus was achieved. A score was calculated based of the percentage of positive tumor cells (<10% = 1; 11–50% = 2; 51–80% = 3; >80% = 4) multiplied by the staining intensity (negative = 0;

weak = 1; moderate = 2; strong = 3). For the immunoreactive score (IRS) the scores for the percentage of positive cells and the staining intensity were multiplied, resulting in a value between 0 and 12. For immunohistochemical quantification, mean differences between groups were compared by one-way analysis of variance with Scheffe multiple comparison tests. The P-value chosen for significance in this study was 0.05.

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Author Contributions

Conceived and designed the experiments: RWS. Performed the experiments: K WY EWJ ARB XC RWS. Analyzed the data: K WY ARB JFF AN XC LJH RMM ARS RWS. Contributed reagents/materials/analysis tools: SHW. Wrote the paper: K WY RWS. Supported the project and contributed conceptually: SHW.

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